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CHEMICALLY MODIFIED LIPOLYTIC ENZYME

FIELD OF THE INVENTION

The present invention relates to a chemically modified lipolytic enzyme, its preparation and its use in detergents. More particularly, it relates to a modified enzyme which shows a first-wash effect.

BACKGROUND OF THE INVENTION

For a number of years, lipolytic enzymes have been used as detergent enzymes to remove lipid or fatty stains from clothes and other textiles, particularly a lipase derived from *Humicola lanuginosa* (EP 258 068 and EP 305 216) sold under the tradename Lipolase ® (product of Novo Nordisk A/S).

A general drawback of detergent lipolytic enzymes is that they exert the best fat removing effect after more than one wash cycle, presumably because the known lipolytic enzymes, when deposited on the fatty stain to be removed, are more active during a certain period of the drying process than during the wash process itself (Gormsen et al., in Proceedings of the 3rd World Conference on Detergents, AOCS press, 1993, pp 198-203). This has the practical consequence that at least two wash cycles (separated by a sufficient drying period) are required to obtain a substantial removal of fatty stains.

WO 97/07202 discloses lipase variants with "first wash performance" which are capable of removing substantial amounts of lard from a lard stained swatch in a one-cycle wash. There is an ever existing need for providing novel lipases with improved washing properties in a variety of commercial detergents. The present invention relates to such novel lipases.

Fatty acid-modified lipases and their use in transesterification have been described. M. Murakami et al., JAOCS, 70 (6), 571-574 (1993); K. Green et al., JAOCS, 75 (11), 1519-1526 (1998).

SUMMARY OF THE INVENTION

The inventors have found that an improved first-wash performance can be achieved by using a lipolytic enzyme which is chemically modified with one or more hydrophobic groups. The modified lipolytic enzymes may further provide additional benefits in washing, such as whiteness maintenance and dingy cleanup. Also, the modified lipolytic enzymes may reduce the formation of fatty acids during the drying process with less risk of forming an unpleasant smell.

Accordingly, the invention provides a lipolytic enzyme which is chemically modified with one, two or three hydrophobic groups. The invention also provides a method of preparing a chemically modified lipolytic enzyme, comprising:

a) modifying the amino acid sequence of the lipolytic enzyme so as to change the number and/or positions of lysine or cysteine residues, and

b) covalently linking hydrophobic groups to the lysine or cysteine residues.

Finally, the invention further provides a detergent composition comprising a surfactant and a lipolytic enzyme which is chemically modified with a hydrophobic group.

DETAILED DESCRIPTION OF THE INVENTION

Lipolytic enzyme

The lipolytic enzyme is an enzyme classified under the Enzyme Classification number E.C. 3.1.1.- (Carboxylic Ester Hydrolases) in accordance with the Recommendations (1992) of the International Union of Biochemistry and Molecular Biology (IUBMB). Thus, the lipolytic enzyme exhibits hydrolytic activity towards ester bonds in mono-, di- and triglycerides, phospholipids (all classes), thioesters, cholesterol esters, wax-esters, cutin, suberin, synthetic esters or other lipilds mentioned in the context of E.C. 3.1.1. Thus, the lipolytic enzyme may, e.g., be what has conventionally been termed a lipase (with triglycerides as substrate), a phospholipase (type A_1, A_2 or B), an esterase or a cutinase.

The lipolytic enzyme may be a microbial lipase, e.g. from bacteria or fungi such as *Humicola* or *Pseudomonas*, particularly lipase from *H. lanuginosa* (this lipase being referred to as Lipolase). The lipolytic enzyme maybe native to such source, or it

may be A variant thereof obtained by altering the amino acid sequence. Examples of such variants are those described in WO 92/05249, WO 94/25577, WO 95/22615, WO 97/04079 and WO 97/07202, WO 98/08939, PCT/DK 99/00068, EP 99610010.3 and Danish patent application PA 1999 00441. A specific example of a variant is the lipase from *Humicola lanuginosa* strain DSM 4109 having the mutations E1SPPCGRRP, E99N, N101S, E239C, Q249R.

The phospholipase may have A_1 or A_2 activity to remove fatty acid from the phospholipid and form a lyso-phospholipid, or it may be have phospholipase B or lysophospholipase activity. It may or may not have lipase activity, i.e. activity on triglycerides. The phospholipase may be of animal origin, e.g. from pancreas (e.g. bovine or porcine pancreas), snake venom or bee venom. Alternatively, the phospholipase may be of microbial origin, e.g. from filamentous fungi, yeast or bacteria, such as the genus *Aspergillus, Fusarium* or *Hyphozyma* (WO 98/18912), particularly the species *A. niger* or *F. oxysporum* (WO 98/26057).

Other examples of lipolytic enzymes are described in Danish patent application PA 1998 01572.

Hydrophobic group

The hydrophobic group may be a fatty acyl group, preferably having 16-20 carbon atoms, straight-chain or branched, saturated, mono- or polyunsaturated, optionally substituted. Examples are palmitoyl (hexadecanoyl), stearoyl (octadecanoyl) and arachoyl (eicosanoyl).

Other examples of hydrophobic groups are those commonly found in surfactants, e.g. a hydrophobic group of non-hydrocarbon origin.

The hydrophobic group(s) is/are preferably attached to the lipid contact zone of the lipolytic enzyme (as described in WO 92/05249) or within 5 Å from the edge of said zone.

Chemical modification

The lipolytic enzyme may be modified by covalently linking one or more hydrophobic groups to lysine or cysteine residues. This can be done by conventional methods, e.g. through a reactive intermediate, e.g. an N-hydroxy-succinimide ester.

Amino acid sequence

The lipolytic enzyme preferably has an amino acid sequence having one, two or three residues of lysine or cysteine, having the hydrophobic group(s) attached to one or more of these residues. This may be achieved by modifying the amino acid sequence through one or more insertions, deletions and/or substitutions by conventional methods. Modification of the amino acid sequence provides a convenient method of adjusting the number and location of the hydrophobic group(s).

The number of other charged amino acids may be changed so as to adjust the isoelectric point. Thus, if the number of lysine residues is decreased (in order to reduce the number of hydrophobic groups), the number of other positively charged amino acids (histidine and/or arginine) may be increased to compensate.

Detergent additive

According to the invention, the lipase may typically be used as an additive in a detergent composition. This additive is conveniently formulated as a non-dusting granulate, a stabilized liquid, a slurry or a protected enzyme. The additive may be prepared by methods known in the art.

DETERGENT COMPOSITION

The detergent compositions of the invention may for example, be formulated as hand and machine laundry detergent compositions including laundry additive compositions and compositions suitable for use in the pretreatment of stained fabrics, rinse added fabric softener compositions, and compositions for use in general household hard surface cleaning operations and dishwashing operations.

The detergent composition of the invention comprises the lipase of the invention and a surfactant. Additionally, it may optionally comprise a builder, another enzyme, a suds suppresser, a softening agent, a dye-transfer inhibiting agent and other components conventionally used in detergents such as soil-suspending agents, soil-releasing agents, optical brighteners, abrasives, bactericides, tarnish inhibitors, coloring agents, and/or encapsulated or non-encapsulated perfumes.

The detergent composition according to the invention can be in liquid, paste, gels, bars or granular forms. The pH (measured in aqueous solution at use con-

centration) will usually be neutral or alkaline, e.g. in the range of 7-11, particularly 9-11. Granular compositions according to the present invention can also be in "compact form", i.e. they may have a relatively higher density than conventional granular detergents, i.e. form 550 to 950 g/l.

The lipase of the invention, or optionally another enzyme incorporated in the detergent composition, is normally incorporated in the detergent composition at a level from 0.00001% to 2% of enzyme protein by weight of the composition, preferably at a level from 0.0001% to 1% of enzyme protein by weight of the composition, more preferably at a level from 0.001% to 0.5% of enzyme protein by weight of the composition, even more preferably at a level from 0.01% to 0.2% of enzyme protein by weight of the composition.

The detergent composition of the invention may comprise the lipase in an amount corresponding to 10-50,000 LU per gram of detergent, preferably 20-5,000 LU/g, e.g. 100-1000 LU/g. The detergent may be dissolved in water to produce a wash liquor containing lipolytic enzyme in an amount corresponding to 25-15,000 LU per liter of wash liquor, particularly 100 - 5000 LU/l, e.g. 300-2000 LU/l. The amount of lipase protein may be 0.001-10 mg per gram of detergent or 0.001-100 mg per liter of wash liquor.

More specifically, the lipase of the invention may be incorporated in the detergent compositions described in WO 97/04079, WO 97/07202, WO 97/41212, PCT/DK WO 98/08939 and WO 97/43375.

Surfactant system

The surfactant system may comprise nonionic, anionic, cationic, ampholytic, and/or zwitterionic surfactants. As described above, the lipase variants of the invention are particularly suited for detergents comprising of a combination of anionic and nonionic surfactant with 70-100 % by weight of anionic surfactant and 0-30 % by weight of nonionic, particularly 80-100 % of anionic surfactant and 0-20 % nonionic. As further described, some preferred lipases of the invention are also suited for detergents comprising 40-70 % anionic and 30-60 % non-ionic surfactant.

The surfactant is typically present at a level from 0.1% to 60% by weight, e.g. 1% to 40%, particularly 10-40 %. preferably from about 3% to about 20% by weight. Some examples of surfactants are described below.

Anionic surfactants

Preferred anionic surfactants include alkyl sulfate, alkyl ethoxy sulfate, linear alkyl benzene sulfonate and mixtures of these.

The alkyl sulfate surfactants are water soluble salts or acids of the formula $ROSO_3M$ wherein R preferably is a C_{10}-C_{24} hydrocarbyl, preferably an alkyl or hydroxyalkyl having a C_{10}-C_{20} alkyl component, more preferably a C_{12}-C_{18} alkyl or hydroxyalkyl, and M is H or a cation, e.g., an alkali metal cation (e.g. sodium, potassium, lithium), or ammonium or substituted ammonium.

Alkylbenzene sulfonates are suitable, especially linear (straight-chain) alkyl benzene sulfonates (LAS) wherein the alkyl group preferably contains from 10 to 18 carbon atoms.

Suitable anionic surfactants include alkyl alkoxylated sulfates which are water soluble salts or acids of the formula $RO(A)_mSO_3M$ wherein R is an unsubstituted C_{10}-C_{24} alkyl or hydroxyalkyl group having a C_{10}-C_{24} alkyl component, preferably a C_{12}-C_{20} alkyl or hydroxyalkyl, more preferably C_{12}-C_{18} alkyl or hydroxyalkyl, A is an ethoxy or propoxy unit, m is greater than zero, typically between about 0.5 and about 6, more preferably between about 0.5 and about 3, and M is H or a cation which can be, for example, a metal cation (e.g., sodium, potassium, lithium, calcium, magnesium, etc.), ammonium or substituted-ammonium cation. Alkyl ethoxylated sulfates as well as alkyl propoxylated sulfates are contemplated herein. Specific examples of substituted ammonium cations include methyl-, dimethyl, trimethyl-ammonium cations and quaternary ammonium cations such as tetramethyl-ammonium and dimethyl piperdinium cations and those derived from alkylamines such as ethylamine, diethylamine, triethylamine, mixtures thereof, and the like.

Other anionic surfactants include salts (including, for example, sodium, potassium, ammonium, and substituted ammonium salts such as mono- di- and triethanolamine salts) of soap, C_8-C_{22} primary or secondary alkanesulfonates, C_8-C_{24} olefinsul-

fonates, sulfonated polycarboxylic acids prepared by sulfonation of the pyrolyzed product of alkaline earth metal citrates.

Nonionic surfactant

The surfactant may comprise polyalkylene oxide (e.g. polyethylene oxide) condensates of alkyl phenols. The alkyl group may contain from about 6 to about 14 carbon atoms, in a straight chain or branched-chain. The ethylene oxide may be present in an amount equal to from about 2 to about 25 moles per mole of alkyl phenol.

The surfactant may also comprise condensation products of primary and secondary aliphatic alcohols with about 1 to about 25 moles of ethylene oxide. The alkyl chain of the aliphatic alcohol can either be straight or branched, and generally contains from about 8 to about 22 carbon atoms.

Further, the nonionic surfactant may comprise polyethylene oxide condensates of alkyl phenols, condensation products of primary and secondary aliphatic alcohols with from about 1 to about 25 moles of ethylene oxide, alkylpolysaccharides, and mixtures hereof. Most preferred are C_8-C_{14} alkyl phenol ethoxylates having from 3 to 15 ethoxy groups and C_8-C_{18} alcohol ethoxylates (preferably C_{10} avg.) having from 2 to 10 ethoxy groups, and mixtures thereof.

Preferred nonionic surfactants are alcohol ethoxylate, alcohol phenol ethoxylate, polyhydroxy fatty acid amide, alkyl polyglucoside and mixtures of these.

EXAMPLES

Example 1: First-Wash Performance

Modified lipases were prepared by covalently linking tetradecanoyl (C_{14}) and hexadecanoyl (C_{16}) groups, respectively, to Lipolase. It was estimated that fatty acyl groups were nearly quantitatively attached to the 6 lysine residues in Lipolase. The two modified lipases were tested as described below, and unmodified Lipolase was tested for comparison.

A number of variants according to the invention were tested in an anionic detergent. The experimental conditions were as follows:

Equipment: Thermostated Terg-o-tometer

Method: 1 cycle wash followed by line drying.

Wash liquor: 1000 ml per beaker

Swatches: 7 (cotton style # 400) swatches (9*9 cm) per beaker.

Stain: Lard coloured with Sudan red (0,75mg Sudan red/g lard).
250 µl of lard/Sudan red heated to 70°C is applied to the center of each swatch, followed by line-drying overnight.

Water: 8.4° German hardness (°dH), Ca : Mg = 2:1

Detergent: 1.8 g/l commercial detergent (Wisk)

Lipase dosage: as indicated below

Wash time: 20 min.

Temperature: 30°C

Rinse: 15 minutes in running tap water.

Drying: Overnight at room temperature (~ 20°C, 30-40 % RH).

Evaluation: The reflectance was measured at 460 nm in a reflectometer. The results are given as ΔR (delta Reflectance) = reflectance of swatches washed in detergent with lipase minus reflectance of swatches washed in detergent without lipase.

Results:

	Lipase	Dosage, LU/l	ΔR
Reference	Lipolase	1329	0.3
		4011	0.7
Invention	Lipolase modified with C_{14}	1617	1.9
		4880	5.2
	Lipolase modified with C_{16}	1212	2.2
		3658	5.5

The results clearly demonstrate that the modified lipases have an improved first-wash performance.

CLAIMS

1. A lipolytic enzyme which is chemically modified with one, two or three hydrophobic groups.

2. The lipolytic enzyme of claim 1 wherein the hydrophobic group(s) is/are located in the lipid contact zone of the lipolytic enzyme or within 5 from the edge of said zone.

3. The lipolytic enzyme of claim 1 or 2 which has an amino acid sequence having one, two or three lysine residues, and wherein the hydrophobic group(s) is/are attached to lysine residue(s).

4. A method of preparing a chemically modified lipolytic enzyme, comprising:

a) modifying the amino acid sequence of the lipolytic enzyme so as to change the number and/or positions of lysine or cysteine residues, and

b) covalently linking hydrophobic groups to the lysine or cysteine residues.

5. The method of claim 4 wherein the modified amino acid sequence has one, two or three lysine or cysteine residues.

6. The method of claim 4 or 5 wherein the modification of the amino acid sequence comprises reducing the number of lysine residues and increasing the number of histidine or arginine residues.

7. A detergent composition comprising a surfactant and a lipolytic enzyme which is chemically modified with a hydrophobic group.

8. The detergent composition of claim 7 wherein the hydrophobic group is a fatty acyl group, preferably having 16-20 carbon atoms.

9. The detergent composition of claim 8 wherein the fatty acyl group is attached to a lysine or cysteine residue in the lipolytic enzyme.